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The molecular analysis of a case of unknown hemolytic anemia using high-throughput sequencing methods.

Abstract

Hemolytic anemias are a group of diseases that have a common denominator - rapid, intravascular breakdown of erythrocytes caused by their abnormal structure. The most common defects are membranopathies and enzymopathies, characterized by disorders in the organization of the erythrocyte membrane and deficits of enzymes, such as glucose-6-phosphate dehydrogenase, which catalyzes the first reaction of the pentose phosphate pathway. Despite the good knowledge of the molecular basis of many cases of the above-mentioned anemia, new types of anemia are still being diagnosed, requiring detailed genetic diagnosis for proper therapy. In the presented thesis an attempt was made to determine the molecular basis of hemolytic anemia, whose phenotype does not correspond to any described so far in the literature.

Erythrocytes, as cells without a nucleus, are not good starting point for molecular analyses, that is why reticulocytes - precursors of erythrocytes, having genetic material in the form of RNA, were selected. Although there are many methods of isolation, they do not provide adequate efficiency when using peripheral blood of healthy people, characterized by a low number of circulating reticulocytes (0.5 - 2.9%). In addition, for the transcriptomic analysis (RNA-Seq), the purity of the material is important, because in this case it translates into the separation of the fraction of nucleated cells, which may be a source of artifacts. Using the previously described techniques, an attempt was made to develop a new method of reticulocyte isolation, based on leukocyte filtration, separation in Ficoll-paque and Percoll gradients, and immunomagnetic separation using a system of microbeads coated with appropriate antibodies. The RNA (cDNA) isolated from the reticulocyte pool was free of transcripts characteristic for nucleated cells as well as from the genomic DNA. Subsequent analysis of high-throughput RNA sequencing data showed downregulation of nine genes, but none of them was found to be associated with the hemolytic anemia phenotype. Due to the fact that it was impossible to select a relatively small pool of transcripts for verification using molecular biology methods, it was decided to perform whole exome sequencing (WES) of patients N61 and N62 with unknown hemolytic anemia. Data obtained using bioinformatics tools showed in patients N61 and N62 a heterozygous mutation in the NT5C3A gene, with

a very low frequency in the population, resulting in a deletion of the phenylalanine residue at position 149 of the amino acid chain of cytosolic 5'-nucleotidase 3A, which is an enzyme involved in the pyrimidine nucleotides metabolism. Genotyping of this change (c. 444_446delGTT) at the mRNA/cDNA level revealed that in the sequence of the 4th transcript variant, characteristic for reticulocytes, this mutation is homozygous in patients and is not detected in both asymptomatic family members as well as controls, and the correct allele is also not detected. Using the Western blot technique, the complete absence of the band characteristic for cytosolic pyrimidine 5'-nucleotidase 3A in the samples of the patients' erythrocyte's cytosol was demonstrated, which is reflected in the data obtained during enzymatic activity measurements (visible lack or minimal catalytic activity towards UMP). Moreover, their ratio of purine to pyrimidine nucleotides is approximately 2.5-fold lower than that of asymptomatic family members and controls, suggesting a complete inhibition of the pyrimidine degradation pathway and its accumulation. In addition, in the WB image, approx. 60% decrease of the amount of the analyzed protein was observed in the N91 father, however his enzymatic activity remains normal. This can be explained by the presence of only one (correct) allele of the NT5C3A gene in the transcriptome of his reticulocytes and a possible unknown mutation in miRNA or transcription factor, which are regulating the expression of the abovementioned gene. Probably the same reason underlies the presence of only the mutant allele detected in the reticulocyte transcriptome of HA patients.

The conducted research failed to explain the presence of only the mutated allele of the NT5C3A gene in the reticulocyte transcriptome of patients N61 and N62, however it has been demonstrated that the deletion of F149 residue in cytosolic pyrimidine 5'-nucleotidase 3A is most likely the direct cause of observed phenotypic effects. Further studies are necessary to understand the molecular mechanism of the NT5C3A abnormal expression.